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HERPES SIMPLEX VIRUS TYPE 2 AND PSEUDORABIES VIRUS ASSOCIATED GROWTH FACTORS AND THEIR ROLE IN THE LATENCY *IN VITRO*

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Summary. – A putative herpes simplex virus type 2 (HSV-2) growth factor (HSGF-2) was detected in a crude extract from virus infected mouse embryo cells. This factor, similar to previously described pseudorabies virus (PRV) associated growth factor (PRGF) was shown to have ability to morphologically transform non-transformed cells and to repress the transformed phenotype of transformed cells. Both activities could be neutralized with two, out of seven monoclonal antibodies directed against glycoprotein B of HSV-2. Both PRGF and HSGF-2 were detected in human embryo lung cells latently infected with PRV or HSV-2 either at 41 °C, or in the presence of phosphonoacetic acid. Human alpha-2 interferon, when present in medium of latently infected cells enhanced the production of both HSGF and PRGF. On the contrary, when latently infected cells were treated with 5-azacytidine the synthesis of both PRGF and HSGF-2 was completely blocked and the virus reactivated from latency replicated to higher titers than in non-treated cells. The role of PRGF and HSGF-2 in the establishment, maintenance and reactivation of latency, as well as in cellular transformation is discussed.

Key words: herpes simplex virus type 2; pseudorabies virus; virus-associated growth factors; latency in vitro

Introduction

The factors controlling latency of some herpes viruses are at present only poorly understood. Many studies have been performed in attempts to mimic latency *in vitro,* however, no latency-associated viral products were demonstrated in these systems (for a review see Rapp, 1984; Stevens, 1989).

Recently a novel putative virus-associated growth factor has been demonstrated in PRV transformed or semipersistently infected human cells (PRGF, Golais

et al., 1988; Golais *et al.*, 1990). PRGF has been shown to have two effects on cultured cells *in vitro*. While normal non-transformed cells, when cultivated in the presence of PRGF acquired the appearence of transformed cells, the phenotype of transformed cells cultivated with PRGF became converted towards the normal one. Phosphonoacetic acid (PAA) and tunicamycin have been shown to inhibit the synthesis of PRGF, indicating that this factor might be a glycosylated viral product synthetized probably late in infection (Golais *et al.*, 1990).

In this paper, the detection and preparation of a similar HSV-2 associated growth factor (HSGF-2) in virus infected mouse embryo (ME) cells is described. HSGF-2 resembles PRGF in several properties, however, at least one resemblance should be stressed. Both growth factors are produced in cells which are non-permissive or semipermissive for replication of corresponding virus. The cells of human origin represent such system for PRV (Golais and Sabó, 1976), and mouse cells for HSV (Szántó *et al.*, 1972). Many *in vitro* systems of herpetic latency have been described under non-permissive conditions for replication of virus (Wigdahl *et al.*, 1982; Russel and Preston, 1986; Cooke and Brown, 1987; Wrzos and Rapp, 1987; Scheck *et al.*, 1989). Therefore the aim of our further study was to determine if some of these replication restricting conditions leading to latency are able to induce the synthesis of PRGF and HSGF-2, and if these virus-associated growth factors could play a role in the establishment, maintenance or reactivation of the latency *in vitro*.

Materials and Methods

Preparation of HSGF-2. The monolayers of ME cells prepared from outbred albino mouse embryos were infected with the Prague strain of HSV-2 at multiplicity of 1 PFU/cell and cultivated at 37 °C. When the cytopathic effect appeared, the cells were trypsinized, pelleted by low speed centrifugation, sonicated and centrifuged for 60 min at 100 000 × g. Virus-free supernatants (crude cell extracts) were lyophilized and applied on to the column of Sephadex G50 (for details see Golais *et al.*, 1990). Column fractions were tested in human embryo lung (HEL) cells for transforming and in HeLa cells for transformed phenotype repressing effect (Golais *et al.*, 1990). After test evaluation, positive 5 ml aliquots of Sephadex G50 fractions were lyophilized, resuspended in 0.2 ml of redistilled water and applied on to column of Sephadex G75. The column (3 × 30 cm) was washed with phosphate buffered saline, pH 7.2 at a constant flow rate 0.2 ml/min. Collected 1 ml samples were evaluated spectrophotometrically and tested again in HEL and HeLa cells. Positive samples were pooled, lyophilized, resuspended in 0.3–0.5 ml of redistilled water, applied on to the Biogel P20 column and separated under the same conditions as with Sephadex G75. Whole separation procedure was also repeated with Sephadex G50 PRGF fractions (Golais *et al.*, 1990). Lyophilized PRGF and HSGF-2 samples of Biogel P20 served as stocks for other studies.

Neutralization of HSGF-2 with antiserum and monoclonal antibodies (MoAbs). Both crude extracts of HSV-2 infected ME cells and their Biogel P20 fractions were incubated at 37 °C with 5 % rabbit antiserum against HSV-1 and HSV-2 and then tested in HEL and HeLa cells (Golais *et al.*, 1988). Furthermore, the Biogel P20 preparations of HSGF-2 were treated with 12 MoAbs against different glycoproteins of both types of HSV (Bystrická *et al.*, 1991). All MoAbs in the form of the ascitic fluid were diluted twofold in serum-free Eagle's basal medium, added to 20 μl HSGF-2 aliquots in an amount of 0.2 and 2 μl and incubated at 37 °C for 90 min. Afterwards 0.1 and 1 μl of each MoAb-

treated sample was added to HeLa cells grown in wells of flat-bottom microplates and 5μl of each sample was added into soft agar layers containing HeLa cells to test the anchorage independent growth (Golais *et al.*, 1990).

HSV-2 and PRV latency in vitro. Tube cultures of HEL cells were infected with the TOP strain of PRV at a multiplicity of 1 PFU/cell or with the Prague strain of HSV-2 at multiplicity of 0.01 PFU/cell and cultivated at 41 °C for 4–5 days. Afterwards, the culture medium (Eagle's basal medium with 5 % heated bovine serum) was changed and the cultivating temperature was shifted down to 37 °C (Kelleher *et al.*, 1975; Golais *et al.*, 1978). After the temperature downshift, part of infected cells received intact medium, other cells infected with HSV-2 or PRV received medium containing human alpha-2 interferon (IFN) (Boehringer), 100 units/ml, or 5-azacytidine (5-azaC) (Sigma), 100 μg/ml. Furthermore, the cells infected with HSV-2 were cultivated in the presence of MoAbs (0.5 % ascitic fluid) against HSV-2, part of HSV-2 infected cells received both MoAbs and IFN. Other HEL cells grown in tubes were pretreated with phosphonoacetic acid (PAA) (Sigma), 250 μg/ml for 24 hr and then similarly infected with PRV and HSV-2. The PAA containing medium was removed after 4–5 days (Colberg-Poley *et al.*, 1979) and the cells were treated similarly as the cells after downshift from 41 °C to 37 °C. Infected cells without previous incubation at 41 °C and without PAA treatment as well as non-infected cells cultivated at both 37 °C and 41 °C served as controls.

Titration of viruses. Virus infectivity was assayed in cells grown in plastic Petri dishes. Tenfold virus dilutions were adsorbed to cells for 90 min at room temperature and then overlaid with Eagle's basal medium containing 2 % heated bovine serum and 0.5 % methylcellulose (Fluka). Three days after inoculation the cells were fixed in 10 % formalin and stained with carbofuchsin. HSV-2 was titrated in HEL and PRV in chick embryo cells. The virus titer were expressed in PFU/ml.

Titration of PRGF and HSGF-2. Freshly trypsinized HeLa cells were dispensed into wells of flat-bottom microplates containing twofold dilutions of PRGF or HSGF-2 samples (about 2×10^4 cells per well) and cultivated for 24 hr at 37 °C. Afterwards the cells were fixed and stained according to Giemsa-Romanowski and observed in microscope for transformed phenotype repressing effect. The endpoint dilution causing the loss of transformed phenotype in 50 % of cultures was considered as one unit of PRGF or HSGF.

Results

Isolation of HSGF-2

Crude extracts from HSV-2 infected ME cells as well as their Sephadex G50 fractions were shown to have an ability to transform HEL cells and to repress the transformed phenotype of HeLa cells (Fig. 1).

Separation of PRGF and HSGF-2 on Sephadex G75 and Biogel P20 proceeded in a similar pattern what indicates that the molecular weight of both factors might be similar (Fig. 2).

Neutralization of HSGF-2

Crude extracts from HSV-2 infected ME cells as well as their Biogel P20 fractions had lost their transforming and transformed phenotype repressing activity when treated with the antiserum against HSV-1 or HSV-2, respectively.

Furthermore, the Biogel P20 preparations of HSGF-2 were treated with 12 MoAbs against different glycoproteins of both types of HSV (Bysrická *et al.*, 1991) and then tested in HeLa cells. Two MoAbs were directed against gB of HSV-2 (gB-2), two MoAbs against gB of HSV-1 (gB-1) and five MoAbs against both types of HSV (gB-1,2). In addition to anti-gB MoAbs, two were directed against gC of HSV-1 (gC-1) and one against gC of HSV-2 (gG-2).

Out of 12 tested MoAbs only two were able to neutralize the transformed

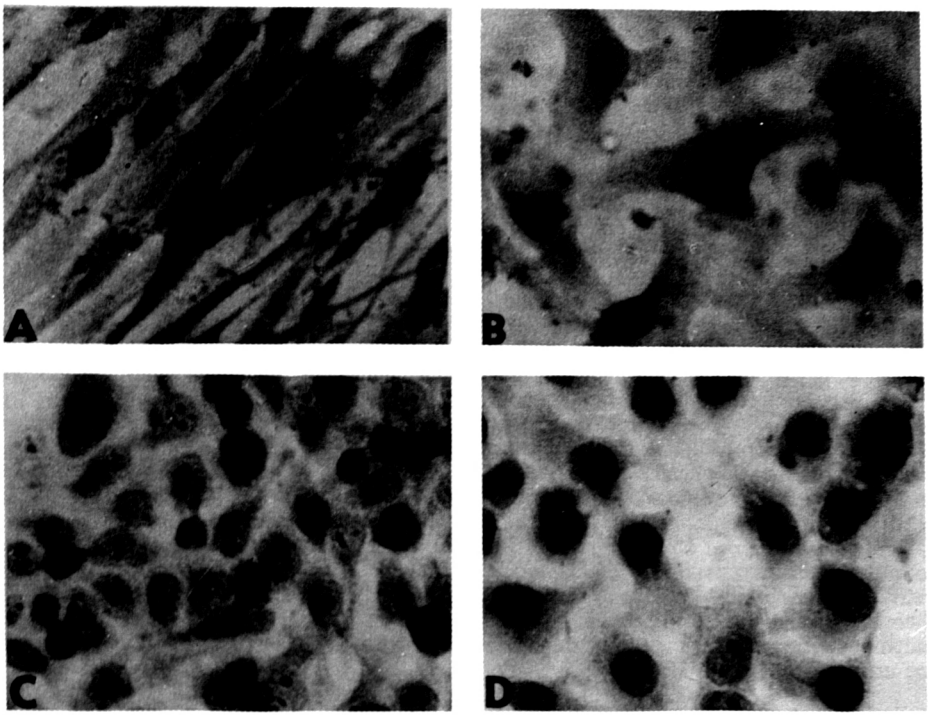

Fig. 1
The effect of HSGF-2 on morphology of HEL and HeLa cells
A: normal HEL cells; B: HEL cells cultivated in the presence of HSGF-2 (Sephadex G50 fraction); C: normal HeLa cells; D: HeLa cells cultivated in the presence of HSGF-2 (Sephadex G50 fraction). Haematoxylin/eosin. Magn. x 850.

phenotype and the anchorage independent growth repressing activity of HSGF-2. One of them, MoAb 499 is directed against gB-2 and the second, MoAb 170 against gB-1,2. The results are summarized in Table 1.

The production of HSGF-2 and PRGF during the latency in vitro

Cultivation of infected HEL cells at 37 °C resulted in appearance of a complete CPE within 2–3 days in contrary to the cells cultivated at 41 °C. As long as the cells were kept at 41 °C no CPE appeared and no virus could be detected in the culture medium. Following transfer to 37 °C, the CPE generally appeared and the infectious virus could be detected in the medium after a latent period of 3–5 days as reported previously (Kelleher *et al.*, 1975; Golais *et al.*, 1978). Virus growth studies were not performed in detail in these experiments, only media of latently infected cells were titrated for the presence of PRGF or HSGF-2.

PRV, in contrary to HSV-2 produced PRGF both at 37 °C and 41 °C, the titers

Table 1. Neutralization of HSGF-2 with MoAbs directed against glycoproteins of HSV-1 and HSV-2

MoAb	Glycoprotein	Virus neutralization		HSGF-2 neutralization
		HSV-1	HSV-2	
733	gB1	-	-	-
740	gB1	-	-	-
201	gB2	-	+	-
499	gB2	-	+	+
144	gB1.2	-	-	-
159	gb1.2	+	+	-
170	gB1.2	-	-	+
T63	gB1.2	-	-	-
T111	gB1.2	-	-	-
809	gC1	-	-	-
303	gG2	-	-	-

at 37 °C being higher than those at 41 °C (Fig. 3A). HSV-2 produced HSGF-2 only during incubation at 41 °C. The preparation of cell extracts from latently infected cells by sonication and ultracentrifugation was not necessary, as both growth factors were shown to be shed into the medium of infected cells. When such media were treated with corresponding antiserum or MoAb, their transforming and transformed phenotype repressing activity disappeared. Both activities were found in the effluent, when the media of latently infected cells were lyophilized and applied to the Sephadex G50 column. On the other hand no activity was demonstrated in non-infected cells cultivated at 41 °C or transferred from 41 °C to 37 °C.

The production of both PRGF and HSGF-2 was considerably enhanced when cultivation temperature was shifted down to 37 °C. A treatment of cells with IFN resulted in a further enhancement of PRGF and HSGF-2 production. When HSV-2 infected cells transferred to 37 °C were cultivated in the presence of MoAb 159, or 201 which neutralize HSV-2 but not HSGF-2, the CPE appeared 1–2 days later and HSGF-2 titers were slightly higher as compared to cells cultivated without MoAbs. A combined treatment with both MoAbs and IFN resulted in a longer delay in an appearance of the CPE (7–10 days) and highest titers of HSGF-2 (Fig. 3B).

No PRGF and HSGF-2 and little or no virus in the absence of CPE were detected in HEL cells cultivated in the presence of PAA. The presence of both growth factors was detectable 24 hr after PAA removal and their production could be similarly enhanced by IFN and MoAbs (data not shown). The appearance of the CPE and the reactivation of virus growth could be detected after a latent period of 3–6 days following PAA removal.

The effect of MoAb against HSGF-2

The latency reactivation did not substantially differ from controls when HSV-2 infected cells were cultivated in the presence of MoAb 170 after temperature downshift or PAA removal. This MoAb specifically reacting with HSGF-2 without neutralizing HSV-2 (see Table 1) had no influence on the length of the latent period or on the replication of the virus reactivated from

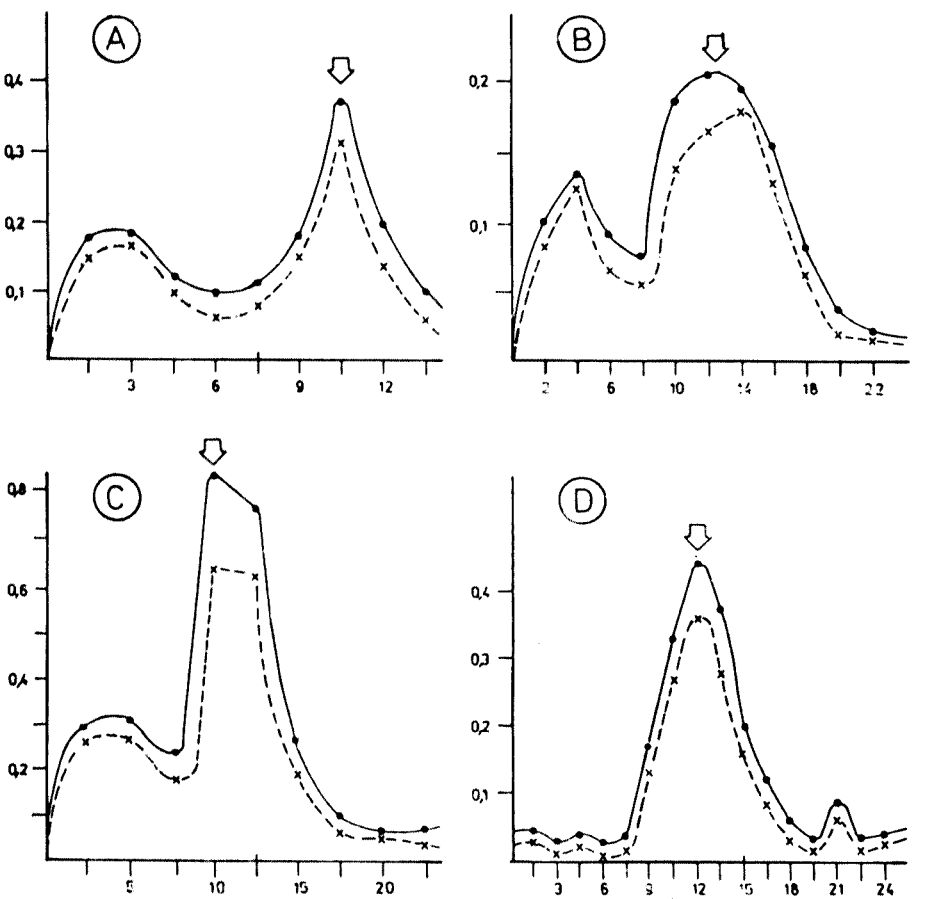

Fig. 2

Separation of PRGF and HSGF-2 by gel chromatography

A - PRGF on Sephadex G75, B - PRGF on Biogel P20, C - HSGF-2 on Sephadex G75, D - HSGF-2 on Biogel P20. The peaks representing PRGF and HSGF-2 are indicated by the arrows. Ordinate: A_{260} (●), A_{280} (x). Abscissa: volume (ml).

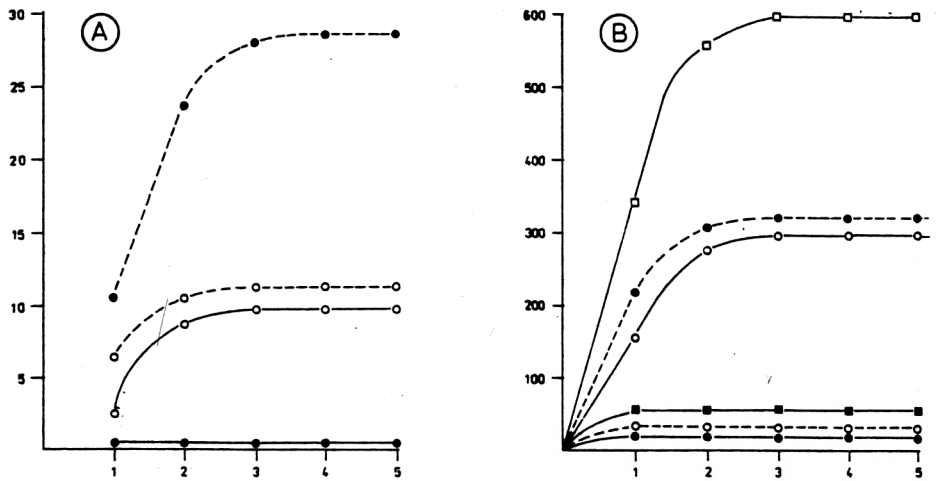

Fig. 3
Production of HSGF-2 and PRGF in HEL cells infected with HSV-2 and PRV
A - infected cells at 37 °C and 41 °C. ●——● HSV2, 37 °C; ○——○ HSV-2, 41 °C; ●- - -● PRV, 37 °C; ○- - -○ PRV, 41 °C. B - infected cells after the downshift from 41 °C to 37 °C. ●——● HSV-2 in an intact medium; ■——■ HSV-2 in an anti-HSV-2 MoAbs containing medium; ○——○ HSV-2 in a medium containing IFN; □——□ HSV-2 in a medium containing anti-HSV-2 MoAbs and IFN, ○- - -○ PRV in an intact medium; ●- - -● PRV in a medium containing IFN. Ordinate: PRGF or HSGF units/ml x 10^3. Abscissa: days after infection.

latency (Fig. 4A). The media of latently infected cells cultivated in the presence of this MoAb were without transforming and transformed phenotype repressing activity.

The effect of 5-azaC on PRGF and HSGF-2 synthesis and on the reactivation of latency

The production of PRGF and HSGF-2 was completely blocked, when 5-azaC was present in the medium of latently infected HEL cells after temperature downshift or after PAA removal. The absence of PRGF or HSGF-2 synthesis caused by 5-azaC had no effect on the length of the latent period, however, the replication of reactivated virus proceeded more rapidly and higher virus titers were obtained than in cells not treated with 5-azaC. The same results were obtained when 5-azaC was presented not only after the downshift from 41 °C but also during incubation at 41 °C. 5-azaC had no effect on establishment of the latency at 41 °C (Fig. 4A). Similar enhancing effect of 5-azaC on PRV replication was observed in HEL cells at 37 °C (Fig. 4B).

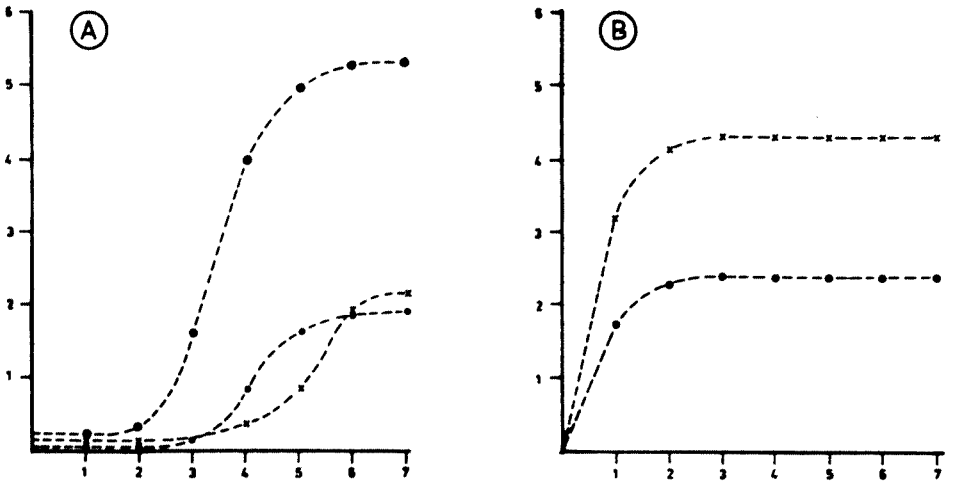

Fig. 4
The effect of MoAb against HSGF-2 and 5-azaC on virus replication
A - HSV-2 after reactivation of latency. HSV-2 in an intact medium (○), HSV-2 in a medium containing MoAb against HSGF-2 (x), HSV-2 in a medium containing 5-azaC (●). B - the effect of 5-azaC on replication of PRV in HEL cells. Control cells (●), cells in the presence of 5-azaC (x). Ordinate: virus titer (log_{10} PFU/ml). Abscissa: days after infection.

Discussion

PRGF, a novel putative viral growth factor was detected in PRV infected and PRV transformed cells (Golais *et al.*, 1988). This factor was shown to have the ability to transform morphologically non-transformed cells and to repress the transformed phenotype of transformed cells. In this paper another putative viral growth factor, HSGF-2, possessing similar properties and probably encoded by HSV-2 genome has been described. Since the synthesis of PRGF could be inhibited by tunicamycin (Golais *et al.*, 1990), which is an inhibitor of glycosylation (Norrild and Pedersen, 1982), it was expected, that these two factors might be glycosylated. This assumption has been confirmed by neutralization of HSGF-2 with 12 MoAbs against some glycoproteins of both types of HSV. It follows from these studies that the gene for gB-2 might be involved in the synthesis of HSGF-2 and the same might be true for a gII gene of PRV (Robbins *et al.*, 1987), which might code for PRGF. The gB gene of HSV codes for a 110–120 K glycoprotein (Spear, 1985), which controls adsorption and penetration of virus with at least two other viral glycoproteins (Spear *et al.*, 1989). According to our results shown in this paper, HSGF-2 might be a cleavage product of gB-2 or a cellular growth factor with a short gB-2 gene-encoded

protein sequence. Some data in literature might support the second possibility. Vaccinia virus was shown to code for growth factor which is related to the epidermal growth factor (EGF) and the alpha-transforming growth factor. This virus-encoded growth factor, different from its cellular counterparts is glycosylated and could be neutralized with both antiviral and anti-EGF antiserum (Stroobant *et al.*, 1985). Porter and Archard (1987) suggested a similar growth factor encoded by Molluscum contagiosum virus. Further studies are now in progress to shed more light on this interesting phenomenon.

HSGF-2 was detected during the latent infection in HSV-2 infected HEL cells at 41 °C and after transfer of these cells to 37 °C. No HSGF-2 was detected in infected HEL cells at 37 °C. On the other hand, PRV infected cells yielded PRGF both at 37 °C and 41 °C, and PRGF titers at 37 °C were considerably higher than those obtained at 41 °C. Similarly, HSGF-2 could be obtained from HSV-2 infected ME cells at 37 °C. Human cells represent a non-permissive or semipermissive system for replication of PRV (Golais and Sabó, 1976), and mouse cells represent a similar system for HSV (Szántó *et al.*, 1972).

As concluded, a PRV and HSV-2 latency *in vitro* established at 41 °C or in the presence of PAA was shown to be associated with the production of PRGF or HSGF-2, however, some cells have a propensity to synthetize such growth factors not only during latency *in vitro* but also in normal cultivating conditions. In such cells a limited or abortive replication of virus is observed (Golais and Sabó, 1976). It is possible that some factors or conditions supporting the PRGF or HSGF-2 synthesis induced during the establishment and maintenance of the latency *in vitro* normally occur in some non-permissive or semipermissive cells. As shown in Fig. 4A, HSGF-2 interfered with the replication of HSV-2 reactivated from the latent state. A virus normally reaching high titers (10^7–10^8 PFU/ml) in HEL cells within 2–3 days replicated very poorly and the spread of the CPE was limited after reactivation of the latency. The virus titers estimated 3–6 days after the reactivation of the virus growth were only about 10^2 PFU/ml. Limited replication of PRV in human cells and HSV in mouse cells might be similarly brought about by virus associated growth factors produced in these cells.

MoAb 170 neutralizing HSGF-2 activity had no effect either on the length of the latent period or on the replication of reactivated virus, indicating that the intracellular HSGF-2 and not that released into the medium is responsible for this interference.

An interesting phenomenon was observed in cells treated with 5-azaC. This inhibitor of methylation (Whitby *et al.*, 1987) had no effect on the length of the latent period, however, it had stimulating effect on replication of reactivated virus. Enhanced viral replication was probably due to the absence of HSGF-2 synthesis brought about by 5-azaC.

Another interesting phenomenon was observed when IFN was present in medium of latently infected cells. IFN was found to enhance the synthesis of both PRGF and HSGF-2. Further enhancement of HSGF-2 production was observed when IFN was combined with two MoAbs neutralizing HSV-2. As

shown in our previous study, prolonged incubation of PRV infected human cells in the presence of antiviral IgG and IFN resulted in morphological transformation of these cells from which a stable transformed H-PR-1 cell line could be derived (Golais *et al.*, 1985), which was shown to produce PRGF. PRGF itself is able to convert normal cells into the transformed ones (Golais *et al.*, 1988; Golais *et al.*, 1990), and the same has been shown with HSGF-2 in this study.

An attractive hypothesis could be, that HSGF-2 and PRGF might be viral oncoproteins synthesized under conditions when production of infectious virus is reduced, inhibited or abortive. Such conditions either exist in some non-permissive or semipermissive cells, or may be induced e. g. during the latent infection. However, an unanswered question remains, why these factors transform normal cells and repress the transformed phenotype of transformed cells. According to the results presented in this paper, both putative viral growth factors do not appear to be directly associated with the establishment, maintenance or reactivation of latency *in vitro*, as 5-azaC is able to stop their synthesis without having an influence on the course of the latency. They may only indirectly influence the course of virus reactivation, inhibiting the replication of reactivated virus.

As already mentioned, the HSV-2 gene for gB appears to be involved in the synthesis of HSGF-2. The expression of this gene might be modified under certain circumstances, so that the synthesis of HSGF-2 and an abortive or limited replication of virus takes place, whereas the gene(s) responsible for normal productive replication might be supressed by methylation. Therefore, the hypomethylating agent 5-azaC might stop the synthesis of HSGF-2 and activate the normal replication cycle of the virus. Similar circumstances might occur in some PRV infected cells. The cells in which PRGF, HSGF-2 or similar virus associated growth factors appear might possess a tendency to be transformed, especially, when antiviral antibodies and IFN act as additional factors.

At present there is no direct evidence supporting these hypotheses, however, the studies concerning this problem are very tempting, because they may elucidate some conditions leading to cellular transformation by some herpesviruses.

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